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A COMPARISON OF *IN VITRO* METHODS FOR ASSESSING THE POTENCY OF THERAPEUTIC ANTISERA AGAINST THE VENOM OF THE CORAL SNAKE *MICRURUS NIGROCINCTUS*

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A. Alape-Girón, K. Miranda-Arrieta, X. Cortes-Bratti, B. G. Stiles and J. M. Gutiérrez. A comparison of *in vitro* methods for assessing the potency of therapeutic antisera against the venom of the coral snake *Micrurus nigrocinctus*. *Toxicon* **35**, 573–581, 1997.—Therapeutic antisera against *Micrurus nigrocinctus* venom were tested for protection against lethality, as well as for inhibition of the nicotinic acetylcholine receptor (AchR)-binding and neutralization of phospholipase A_2 (PLA_2) activities of the homologous venom. Protection against venom lethality did not correlate with inhibition of AchR-binding activity, whereas there was a significant correlation between antisera potency and inhibition of PLA_2 activity ($r = 0.82$, $n = 10$, $P < 0.02$). Inhibition of PLA_2 activity could be useful in assessing the protective efficacy of *M. nigrocinctus* antisera during antivenom production. *Micrurus nigrocinctus nigrocinctus* venom proteins were fractionated by cation-exchange chromatography on Mono S FPLC and fractions assayed for lethality, AchR-binding and PLA_2 activities. Antisera were titrated by enzyme-linked immunoassay (ELISA) against a crude *M. n. nigrocinctus* venom, two FPLC lethal fractions containing AchR-binding activity, and two toxins purified from *M. n. nigrocinctus* venom. No correlation was found between protective efficacy and the ELISA titer against any of these antigens. Compared to other elapid venoms that contain few toxins as major components, *M. n. nigrocinctus* venom appears to be more complex and its lethal effect is likely to be due to the combined effect of several neurotoxins.

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INTRODUCTION

Micrurus snakes (New World coral snakes) are widely distributed from the south-eastern United States to Argentina (Campbell and Lamar, 1989). Severe envenomations by *Micrurus* snakes result in neurotoxic symptoms and require serotherapy (Russell, 1983; Kitchens and Van Mierop, 1987). Several laboratories in different countries produce therapeutic antivenoms by hyperimmunizing animals with crude venom from one or several *Micrurus* species (Theakston and Warrell, 1991; Gutiérrez *et al.*, 1991; Rawat *et al.*, 1994). Although mouse lethality assays are needed in the final testing of a given antivenom batch (WHO, 1981), more simple *in vitro* techniques are desirable for assessment of antivenom potency at earlier stages of antivenom preparation.

The use of large antivenom volumes during serotherapy of snakebite envenomations increases the risk of serious side-effects (Sullivan, 1987). If animals were immunized only with the toxic venom components, the resulting antivenoms would probably be more efficacious, enabling the use of smaller volumes for serotherapy (Ménez, 1991). Thus it would be desirable to identify and purify the most important toxins from a venom in order to prepare novel antigenic mixtures for the production of more efficacious antivenoms.

Micrurus nigrocinctus is the most abundant, medically important coral snake in Central America (Alape-Girón *et al.*, 1994a). The mouse LD_{50} of *Micrurus nigrocinctus nigrocinctus* venom is 0.765 mg/kg i.p. and 2 mg/kg s.c. (Gutiérrez *et al.*, 1991). In the mouse phrenic nerve–diaphragm, this venom induces electrophysiological and ultrastructural changes that suggest the presence of postsynaptically and presynaptically acting toxins (Goularte *et al.*, 1995, 1996). We previously found that some components of *M. n. nigrocinctus* venom bind to the nicotinic acetylcholine receptor (AchR), and that antivenom inhibits the binding of homologous and heterologous α-neurotoxins to AchR (Alape-Girón *et al.*, 1996a,b).

The aims of this study were to: (1) determine whether the potency of *M. nigrocinctus* antisera correlates with inhibition of AchR-binding and PLA_2 activities found in the homologous venom; (2) identify the lethal fractions of *M. n. nigrocinctus* venom; and (3) determine whether the protective efficacy of antisera correlates with the enzyme-linked immunosorbent assay (ELISA) titer against crude *M. n. nigrocinctus* venom, lethal fractions, or purified toxins.

MATERIALS AND METHODS

Venoms and toxins

A pool of *M. n. nigrocinctus* venom was obtained from more than 100 specimens collected in the Pacific region of Costa Rica. *Naja naja oxiana* neurotoxin II (NT II) and *Bungarus multicinctus* α-bungarotoxin were from Sigma (St Louis, MO, U.S.A.). *Notechis scutatus scutatus* notexin was kindly provided by Dr Bertil Persson (Uppsala University, Sweden). Mnn-4 and nigroxin B were purified from *M. n. nigrocinctus* venom as described elsewhere (Alape-Girón *et al.*, 1996b).

Antisera

Micrurus nigrocinctus antivenoms (batches 207, 221 and 232) were from Instituto Clodomiro Picado (Gutiérrez *et al.*, 1991). Horse antisera against *M. nigrocinctus* venom were produced as described by Bolaños and Cerdas (1980). Antiserum to *N. s. scutatus* notexin was prepared in rabbits (Alape-Girón *et al.*, 1996b). Antiserum to *N. n. oxiana* neurotoxin II (NT II) was prepared in rabbits (2 kg body weight) by s.c. injection of native toxin (12.5 μg) emulsified in Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.). Boosters were given s.c., three in Freund's complete adjuvant and eight in Freund's incomplete adjuvant, according to this scheme: days 12 and 29: 12.5 μg; days 36, 43, 50 and 57: 25 μg; days 67, 74 and 81: 30 μg; with bleeding on day 85.

FPLC fractionation of M. n. nigrocinctus *venom proteins*

Micrurus nigrocinctus nigrocinctus venom samples dissolved in 1.5 ml of 0.05 M HEPES buffer, pH 8, were separated on a Mono-S HR 16/10 column using a Pharmacia FPLC instrument, a linear gradient of NaCl and a flow rate of 3 ml/min. Sixty-four fractions, 3 ml each, were collected and protein concentrations measured by the Bradford assay (Spector, 1978) using bovine serum albumin as a standard.

Lethality, AchR-binding, and PLA_2 activities of venom fractions

Lethality of the material corresponding to the separated peaks was determined by i.p. injection [1 μg protein in 0.5 ml of phosphate-buffered saline, pH 7.2 (PBS)] into groups of four to eight Swiss–Webster mice (16–18 g). Deaths were recorded up to 72 hr.

AchR-binding activity and competitive assays were performed as described previously (Stiles, 1991; Alape-Girón *et al.*, 1996b). In brief, 100 μl of purified AchR (50 μg/ml) was incubated with varying amounts of *N. n. oxiana* NT II for 20 min at 22°C and the mixture added to wells coated with venom fractions. After a 1 hr incubation at 22°C, plates were washed three times with PBS containing 0.1% Tween 20 (PBST), and guinea-pig antiserum to AchR [diluted in PBS containing 0.1% Tween-20 and 1% (w/v) gelatin, PBSTG] was put into wells. Following a 1 hr incubation at 22°C and four washes with PBST, goat anti-guinea-pig IgG-alkaline phosphatase conjugated was added for 1 hr at 22°C. Plates were washed five times with PBST, para-nitrophenyl phosphate diluted in diethanolamine buffer, pH 9.8, was added, and absorbances were read at 405 nm after 30 min.

PLA_2 activity was measured using an indirect hemolytic assay (Gutiérrez *et al.*, 1988). Fractions diluted 1:150 in PBS were put into 4 mm wells in agarose gels containing 4% sheep erythrocytes, 4% egg yolk as a source of lecithin, and 10 mM $CaCl_2$. The diameters of hemolytic halos were measured after a 20 hr incubation in a humidified chamber at 37°C. None of the fractions produced direct hemolysis in gels without egg yolk.

In vivo *neutralization assays*

A fixed amount of *M. n. nigrocinctus* venom was incubated with varying amounts of antivenoms for 30 min at 37 C. Each mixture (0.5 ml), containing four LD_{50}s of venom (52 μg), was injected i.p. into groups of four Swiss–Webster mice (16–18 g) and deaths were recorded up to 72 hr. Controls received four LD_{50}s of venom in PBS. Results were analyzed using PROBIT and neutralization was expressed as effective dose 50% (ED_{50}), defined as the volume of antivenom (μl)/mg venom needed to prevent death in 50% of the injected mice.

Inhibition of AchR binding

Inhibition of *M. n. nigrocinctus* venom binding to AchR by antivenoms was measured essentially as described previously (Alape-Girón *et al.*, 1996a). In brief, Immulon II microtiter plates (Dynatech, Chantilly, VA, U.S.A.) were coated overnight at 4°C with *M. n. nigrocinctus* venom (5 μg/well). The remaining binding sites were blocked for 30 min at 37°C with 300 μl of PBS containing 1% (w/v) gelatin (PBSG), and then dilutions of antisera were added and incubated 30 min at 37°C. The AchR, guinea-pig anti-AchR serum, anti-guinea-pig alkaline phosphatase conjugate and para-nitrophenyl phosphate were serially added and absorbances recorded at 405 nm after 30 min. Control wells were incubated with a non-immune horse serum or an antivenom of non-related specificity. Inhibition curves were obtained by plotting the absorbance readings against the logarithm of antivenom dilutions. Results are expressed as effective dilution 50% (ED_{50}), defined as the dilution at which AchR binding was inhibited by 50% compared to wells incubated in parallel with PBSG instead of antiserum.

Inhibition of PLA_2 activity

Inhibition of *M. n. nigrocinctus* PLA_2 activity by antivenoms was measured as described by Gutiérrez *et al.* (1988). In brief, a constant amount of venom was incubated with different dilutions of antivenom for 30 min at 37°C. Then, 10 μl of each mixture (containing 1.2 μg of venom) was added to duplicate wells in agarose–erythrocyte–egg yolk gels. The diameter of the hemolytic halos was measured and inhibition of PLA_2 activity was calculated as a percentage of the hemolysis caused by venom alone. A non-immune horse serum or an antivenom of non-related specificity was used as control. Inhibition of PLA_2 activity was expressed as effective dose 50%, defined as the volume of antivenom (μl) required to reduce by 50% the PLA_2 activity of 1 mg of venom. ED_{50} values were obtained for each antivenom in three independent experiments.

ELISAs

Microtiter plates were coated overnight at 22°C with 0.5 μg protein/well, dissolved in carbonate buffer, pH 9.6. The remaining binding sites were blocked with 150 μl of 2% bovine serum albumin (blocking solution) for 15 min at 37°C. Various dilutions of antivenoms in blocking solution were then added (100 μl/well). After a 1 hr incubation at 37°C and five washes with a Tris 50 mM, NaCl 150 mM, $ZnCl_2$ 0.020 mM, $MgCl_2$ 1 mM buffer, pH 7.4, 100 μl of anti-horse IgG-alkaline phosphatase conjugate (Sigma) was added per well. After a 1 hr

incubation at 37°C, plates were washed five times and 100 μl/well of a para-nitrophenyl phosphate substrate solution (1 mg/ml) diluted in diethanolamine buffer, pH 9.8, was added. Absorbances were recorded at 405 nm after 60 min. Titration curves were obtained by plotting the absorbance readings against the logarithm of antivenom dilutions. The titer was defined as the inverse logarithm of the dilution giving 0.5 absorbance units. Negative controls on each plate were either non-immune horse serum or an antivenom of non-related specificity. Dilutions of a *M. nigrocinctus* antivenom (batch 207) were included on every plate as a reference sample to control plate-to-plate variations.

RESULTS

Neutralization of M. n. nigrocinctus *venom by antivenoms*

The potencies of ten *M. nigrocinctus* antivenom samples to protect against lethality in mice and their abilities to inhibit AchR-binding and PLA_2 activities of the homologous venom are shown in Table 1. Intraperitoneal injection of 4 LD_{50} of venom caused respiratory failure and death within 2 hr. Antivenoms protected against lethality of *M. n. nigrocinctus* venom with ED_{50}s ranging from 2732 to 7519 μl/mg. Antivenoms inhibited the binding of *M. n. nigrocinctus* α-neurotoxins to AchR with ED_{50}s ranging from 217 to 2250 μl/mg, but no correlation was found between the protective efficacy and inhibition of AchR-binding ($r = 0.34$; $P > 0.05$). Antivenoms inhibited the PLA_2 activity with ED_{50}s ranging from 108 to 515 μl/mg, which correlated significantly with protection against lethality ($r = 0.82$; $P < 0.02$).

Lethality of M. n. nigrocinctus *venom fractions*

Micrurus nigrocinctus nigrocinctus venom basic proteins were separated in 19 peaks on Mono S FPLC (Fig. 1). A mixture containing *M. n. nigrocinctus* venom proteins with isoelectric points lower than 8.0 (material not bound to the Mono S) was not lethal when injected i.p. into mice at a dose of 55–62 μg/kg. In contrast, material corresponding to ten of the 19 peaks separated by Mono S (peaks IV, IX, XI–XVII, and XIX, marked by asterisk in Fig. 1) was lethal at that dose. Material corresponding to peak IV caused flaccid

Table 1. Potency of *M. nigrocinctus* antivenoms to protect against lethality of the homologous venom and inhibition of AChR-binding and PLA_2 activities

Antivenom sample no.	Lethality (ED_{50})*	AChR binding (ED_{50})†	PLA_2 activity (ED_{50})‡
1	7519	504	438
2	7042	1435	372
3	6135	475	515
4	6135	217	279
5	5000	689	183
6	5000	n.t.§	280
7	4386	712	214
8	4184	984	108
9	4098	2250	204
10	2732	1004	141

*Amount (μl) of antivenom/mg venom needed to protect half of the mice injected with 4 LD_{50} of venom.

†Dilution at which the AChR-binding activity was inhibited by 50% compared with control wells incubated without antiserum.

‡Amount (μl) of antivenom required to reduce by 50% the PLA_2 activity of 1 mg of venom.

§Not tested.

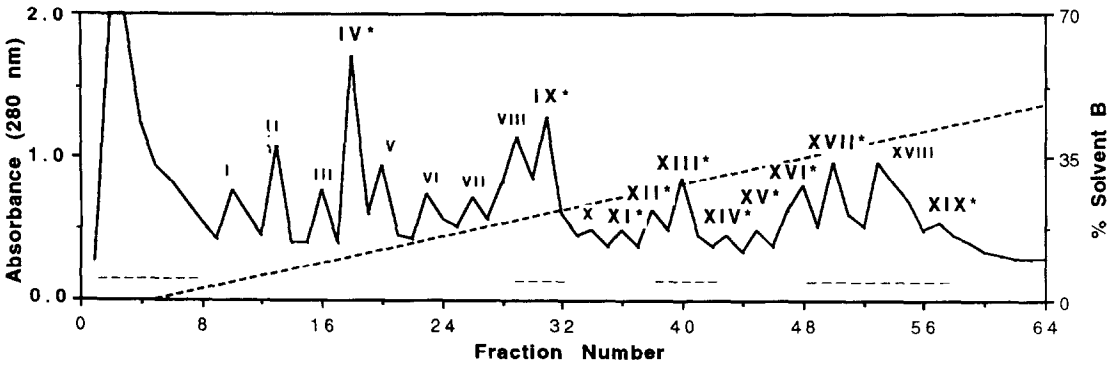

Fig. 1. Elution profile of *M. n. nigrocinctus* venom (120 mg) on Mono S FPLC with a linear gradient of NaCl.
Solvent A: 0.05 M HEPES, pH 8.0; solvent B: solvent A + 1 M NaCl. Gradient: 0–48% B in 60 min. Lethal peaks are noted by asterisks and peaks with PLA_2 activity are indicated by dashed lines at the bottom.

paralyis, breathing difficulties, and death in less than 30 min after injection, whereas time to death caused by the other fractions was longer than 2 hr.

Material corresponding to peaks IV and XVI (fractions 17–19 and 46–49, respectively) was lethal and contained AchR-binding activity that was dose-dependently inhibited by *N. n. oxiana* NT II (Table 2), suggesting that *M. n. nigrocinctus* venom contains several α-neurotoxins with different charges. Moroever, material corresponding to peak IV was recognized in an ELISA by antibodies to *N. n. oxiana* NT II, which also inhibited its binding to AchR (data not shown). These results strongly suggest that peak IV contains short-chain α-neurotoxin(s), which share neutralizing epitope(s) with *N. n. oxiana* NT II.

PLA_2 activity was detected in material corresponding to peaks VIII–IX, XII–XIII and XVI–XIX (Fig. 1) and, furthermore, this material was recognized in an ELISA by antibodies to *N. s. scutatus* notexin (data not shown).

Titration of antivenoms

Micrurus nigrocinctus antivenoms were titrated against one presynaptic toxin, *N. s. scutatus* notexin, and two postsynaptic toxins: a long-chain α-neurotoxin, *B. multicinctus* α-bungarotoxin, and a short-chain α-neurotoxin, *N. n. oxiana* neurotoxin II (data not shown). No correlation was found between the potency of antivenoms to protect against lethality and the ELISA titers against any of those heterologous toxins.

Antivenoms were titrated by ELISA against crude *M. n. nigrocinctus* venom and lethal fractions with AchR-binding activity, corresponding to peaks IV and XVI (Table 3). No significant correlation was found between antivenom potency and ELISA titer against crude venom or any of the AchR-binding fractions. Antivenoms were also titrated against two toxins purified from *M. n. nigrocinctus* venom: Mnn-4, an AchR-binding protein, and nigroxin B, a basic PLA_2 (Table 3). No correlation was found between antivenom potency and the ELISA titer against any of these homologous toxins.

Table 2. AChR-binding activity* of *M. n. nigrocinctus* venom fractions from Fig. 1 in the presence of varying amounts of *N. n. oxiana* NT II

Fraction numbers†	Without competitor	*N. n. oxiana* NT II (0.1 μg/ml)	% Binding	*N. n. oxiana* NT II (1 μg/ml)	% Binding	*N. n. oxiana* NT II (10 μg/ml)	% Binding
Peak IV							
17	1.50 ± 0.04‡	1.41 ± 0.03	94§	0.88 ± 0.04	59	0.09 ± 0.01	6
18	1.46 ± 0.04	1.33 ± 0.01	91	0.82 ± 0.04	56	0.09 ± 0.00	6
19	1.37 ± 0.05	1.26 ± 0.02	92	0.79 ± 0.02	58	0.10 ± 0.00	7
Peak XVI							
46	0.48 ± 0.06	0.45 ± 0.03	94	0.36 ± 0.06	75	0.24 ± 0.02	50
47	0.64 ± 0.05	0.62 ± 0.04	97	0.37 ± 0.01	58	0.16 ± 0.01	25
48	0.85 ± 0.03	0.78 ± 0.01	92	0.61 ± 0.03	72	0.42 ± 0.03	49
49	1.05 ± 0.03	0.98 ± 0.03	93	0.86 ± 0.06	82	0.71 ± 0.02	68
NT II	1.50 ± 0.12	1.29 ± 0.08	86	0.60 ± 0.07	40	0.09 ± 0.01	6

*AchR (with or without *N. n. oxiana* NT II) was added to venom fractions adsorbed on to microtiter wells. Binding was detected by the addition of guinea-pig anti-AchR serum, anti-guinea-pig alkaline phosphatase conjugate and substrate.

†Tube numbers correspond to those indicated in Fig. 1.

‡Absorbance values recorded at 405 nm (mean ± S.D; $n = 3$).

§Binding percentages relative to binding without competitor.

Table 3. ELISA titer of *M. nigrocinctus* antivenoms against the homologous venom, lethal fractions with AChR-binding activity, and two purified toxins

Antivenom sample no.	Titers* to				
	Crude venom	Peak IV	Peak XVI	Mnn-4	Nigroxin B
1	3.2	2.5	2.5	3.4	4.0
2	4.3	3.3	3.6	3.4	4.3
3	4.0	3.6	3.7	3.4	4.9
4	3.1	2.5	2.4	3.0	3.5
5	4.1	3.3	3.4	3.3	4.4
6	n.t.†	n.t.	n.t.	3.5	4.2
7	3.9	3.2	3.4	3.3	4.3
8	3.7	2.9	2.9	3.4	4.0
9	4.1	3.6	3.9	3.2	4.0
10	3.6	2.7	2.6	3.4	3.5

*Negative log of the dilution giving 0.5 absorbance units.
†Not tested.

DISCUSSION

Micrurus nigrocinctus antivenoms contain antibodies which neutralize the lethal effect of the neurotoxins present in the homologous venom, as determined by mouse lethality assays. We previously reported the use of an AchR-binding assay to evaluate the ability of *M. nigrocinctus* antivenoms to inhibit binding of *M. n. nigrocinctus* α-neurotoxins to AchR (Alape-Girón *et al.*, 1996a). In the present work, we found no correlation between antivenom potency and inhibition of *M. n. nigrocinctus* α-neurotoxins binding to AchR. In contrast, antivenom potency correlated significantly with inhibition of PLA_2 activity found in *M. n. nigrocinctus* venom. This finding suggests a role for toxic PLA_2s in *M. n. nigrocinctus* venom lethality. Alternatively, antibodies that inhibit PLA_2s may parallel the presence of antibodies against another lethal toxin(s). Anyhow, inhibition of PLA_2 activity is a simple and inexpensive assay, which could be useful in initial potency assessments of *M. nigrocinctus* antisera, minimizing the need for mouse lethality assays during antivenom production. For antisera to *Crotalus durissus terrificus* venom, there is a good correlation between neutralization of PLA_2 and lethal activities of homologous venom (Da Silva and Bier, 1982). The component responsible for the lethal effect of *C. d. terrificus* venom is crotoxin, a neurotoxic PLA_2 that constitutes 68% of the total venom protein (Hanashiro *et al.*, 1978). This work shows that *M. n. nigrocinctus* venom contains multiple PLA_2s with different charges, that are antigenically related to *N. s. scutatus* notexin, the most toxic monomeric venom PLA_2 (Mollier *et al.*, 1989). Interestingly, *N. s. scutatus* antivenom was reported to protect against the lethal effect of *Micrurus fulvius* venom (Minton, 1967), which is antigenically very similar to that of *M. n. nigrocinctus* (Bolaños *et al.*, 1978; Alape-Girón *et al.*, 1994b). *Micrurus corallinus* and *M. n. nigrocinctus* venoms exert both postsynaptic and presynaptic effects on neuromuscular transmission (Vital-Brazil and Fontana, 1983/84; Goularte *et al.*, 1995). Furthermore, these two venoms induce an increase in the number of omega-shaped axolemmal indentations and a decrease in the number of synaptic vesicles at the motor nerve terminal in mouse phrenic nerve–diaphragm preparations, suggesting that *M. corallinus* and *M. n. nigrocinctus* venoms contain neurotoxic PLA_2s (Cruz-Hofling *et al.*, 1983/84; Goularte *et al.*, 1996). Lethal FPLC

fractions from *M. n. nigrocinctus* venom with PLA_2 activity might contain some of these neurotoxic PLA_2s.

It has been recently suggested that the chick biventer cervicis preparation could be used to assess the protective activity of antivenoms to neutralize the neuromuscular effects of neurotoxic snake venoms (Barfaraz and Harvey, 1994). Since there are both presynaptically and postsynaptically acting toxins in *M. n. nigrocinctus* venom, it would be interesting to test whether there is a correlation between this technique and mouse lethality assays for *M. nigrocinctus* antivenoms.

Theakston and Reid (1979) reported the use of ELISAs with crude venoms as antigens to assess the potency of viperid and elapid antivenoms. More recently, Rungsiwongse and Ratanabanangkoon (1991) suggested that ELISAs using lethal toxin-containing fractions as antigens would provide better estimates of the potency of elapid antivenoms. These authors found a good correlation between the potency of *Naja naja siamensis* antivenoms and the ELISA titer against a fraction containing α-cobratoxin, the major α-neurotoxin of *N. n. siamensis* venom that constitutes 35% of the total venom protein (Karlson *et al.*, 1971). However, in the case of *M. nigrocinctus* antivenoms, we observed no correlation between potency and the ELISA titers against either crude venom, AchR-binding fractions, or purified toxins.

The findings suggest that *M. n. nigrocinctus* venom contains multiple α-neurotoxins and toxic PLA_2 isoforms, but not a single predominant lethal toxin. It is very likely that the lethality of *M. n. nigrocinctus* venom results from the combined action of several neurotoxins. Although *M. nigrocinctus* antivenom potency correlates with the ability to inhibit PLA_2 activity of the homologous venom, further studies will be needed to clarify the role of toxic PLA_2s and α-neurotoxins in the lethal effect of *M. n. nigrocinctus* venom.

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